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Prevalence, distribution and characterisation of ceftiofur resistance in *Salmonella enterica* isolated from animals in the USA from 1999 to 2003

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Abstract

Third-generation cephalosporin (3GC) antimicrobials are the drugs of choice for treatment of salmonellosis in children. *Salmonella* isolated in the USA are assayed by the National Antimicrobial Resistance Monitoring System (NARMS) for resistance to antimicrobials including first-, second- and third-generation cephalosporins. From 1999 to 2003, 34,411 *Salmonella* were isolated from animals in the USA, of which 10.9% were found to be resistant to ceftiofur, a 3GC used in animals, whilst only 0.3% were resistant to ceftriaxone, a 3GC used in human medicine. Ceftiofur resistance rose from 4.0% in 1999 to 18.8% in 2003. Isolates from diagnostic laboratories had higher levels of resistance (18.5%), whereas levels in isolates from on-farm (3.4%) and slaughter (7.1%) sources were lower. Animals with a higher than average proportion of resistant *Salmonella* included cattle (17.6%), horses (19.2%) and dogs (20.8%). Levels in turkeys (6.8%), chickens (7.1%), eggs (3.6%) and swine (4.6%) were lower. Resistance varied between *Salmonella* serotypes. A few serotypes had significantly high levels, e.g. *S.* Newport was 70.4% ceftiofur resistant. Resistance was predominantly associated with bla_{CMY-2}-encoding plasmids. These data suggest that the acquisition of resistance plasmids and the spread of specific serotypes harbouring these plasmids are driving the observed resistance to ceftiofur in *Salmonella* animal isolates.
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Keywords: *Salmonella*; Cephalosporin; Ceftiofur; Antimicrobial resistance; Animal; Plasmid; bla_{CMY-2}

1. Introduction

Development of antimicrobial resistance in bacteria is a serious public health concern. Investigations must be carried out to track and monitor the emergence of resistance. It is generally accepted that the use of antimicrobials in humans and animals has contributed to the development of resistance to these compounds [1,2]. However, the degree of impact that antimicrobial use in animals has on human health is debated.

Monitoring the development of antimicrobial resistance in bacteria isolated from animals as well as humans is necessary to ensure the proper use and prolonged lifespan of current antimicrobials [3–6]. In the USA, the National Antimicrobial Resistance Monitoring System (NARMS), a collaboration between the U.S. Department of Agriculture (USDA), the Centers for Disease Control and Prevention (CDC) and the Food and Drug Administration (FDA) [4,7], was established for this purpose. *Salmonella* is the sentinel organism of the system.

Infection with *Salmonella enterica* can result in a variety of diseases, from gastroenteritis and diarrhoea to enteric fever [8]. *Salmonella* has been isolated from animals, animal products, produce, humans and the environment, and transmission to humans is thought to be from contaminated food [9–11]. The CDC estimates that salmonellosis accounts for

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up to 1.4 million infections each year in the USA, most of which resolve without treatment; however, enteric fever and systemic infections can be life threatening and may require treatment with antibiotics [12]. In the past few decades, development of antimicrobial resistance in *Salmonella* has been reported and appears to be increasing [13,14]. Of global concern is the development of multidrug-resistant (MDR) *Salmonella* as well as resistance to third-generation cephalosporins (3GCs) [15], the antibiotic of choice for treating systemic *Salmonella* infections in children and the elderly [16].

In a study by Gray et al. [17], NARMS isolates collected in 1997 and 1998 were rarely resistant to 3GCs, with ca. 2.0% (112/5709) being resistant to ceftiofur, a 3GC used in animal medicine. Ten *S. enterica* serotypes were found to have significantly more resistant isolates, and isolates from turkeys, horses, cats and dogs were more likely to be resistant. In general, isolates originating from veterinary diagnostic laboratories were more resistant than those collected during inspections at slaughter houses. In Europe, Africa, Asia and South America it has been found that a number of β-lactamase-encoding genes confer resistance to 3GCs [18]. These include variants of bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{AmpC} β-lactamases [19,20]. Interestingly, all ceftiofur-resistant isolates in the 1997–1998 study of US animals carried a plasmid containing the bla_{CMY-2} gene that encodes an AmpC-type β-lactamase [17].

In the current study, *Salmonella* isolated from animal sources from 1999 to 2003 ($n = 34,411$) were analysed for resistance to the 3GC ceftiofur. The prevalence of resistant isolates from specific animal species was analysed as well as the type of sample from which they originated (diagnostic, on-farm or slaughter) to determine their distribution and to identify animals with higher than average levels of resistant *Salmonella*. The serotypes of the isolates were also analysed to identify highly resistant serotypes. The mechanisms responsible for ceftiofur resistance in US animal isolates also underwent molecular analysis to identify resistance genes, to determine their location and to evaluate their transmissibility to other *Salmonella* strains.

2. Materials and methods

2.1. Strains, growth conditions and antimicrobial susceptibility

Salmonella enterica isolates were obtained from the NARMS bacterial collection, from slaughter, diagnostic laboratories or on-farm sites, and isolated as previously described (http://www.cdc.gov/narms/) [21,22]. Susceptibility testing for *Salmonella* was performed using the Sensititre™ system (Trek Diagnostic Systems Inc., Westlake, OH) and included amikacin, amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, cefalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid,

streptomycin, sulfamethoxazole, tetracycline and trimethoprim/sulfamethoxazole. Clinical and Laboratory Standards Institute (CLSI) breakpoints, control strains and guidelines for interpretations were followed [23]. Data are available on the website http://www.ars.usda.gov/Main/docs.htm?docid=6750.

2.2. Statistical analysis

The Pearson correlation coefficient was used to determine whether the change in the percentage of *Salmonella* isolates resistant to ceftiofur increased at a continuous rate from 1997 to 2003. The χ^2 test was used to determine whether *Salmonella* isolated from certain animals were more resistant to ceftiofur than the overall average and whether specific *S. enterica* serotypes were more or less resistant than average. Significance was expressed at the $P < 0.0001$ level.

2.3. Polymerase chain reaction (PCR) analysis

PCR reactions were performed to detect the bla_{CMY-2}, bla_{CTX-M} groups I–IV, bla_{TEM} and bla_{SHV} β-lactamase genes [19,20] as well as the integron genes [24] *intI1*, *intI2*, *intI3* and *intI4*. Primers are shown in Table 1. Assays were performed in a MJ Research PTC-200 DNA Engine thermal cycler (Bio-Rad Laboratories, Waltham, MA) as previously described [17,25,26], except for bla_{TEM} and *intI1–4*, where cycling parameters were 94 °C for 1 min, 46 °C for 1 min and 72 °C for 1 min for 30 cycles for bla_{TEM}, and 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min for 30 cycles for *intI1–4*.

2.4. Plasmid and Southern hybridisation analysis

Plasmid DNA was isolated using the procedure described by Kado and Liu [27]. One microgram of each DNA sample was separated by electrophoresis on a 0.6% agarose gel in Tris-acetate–EDTA buffer at 50 V at 4 °C for 16 h. Comparison with digoxigen (DIG)-labelled lambda *Hind*III ladder (Roche, Indianapolis, IN), supercoiled DNA ladder (Invitrogen, Carlsbad, CA) and control plasmids was used to the estimate size of isolate plasmids. Gels were transferred to a positively charged membrane with a Vacuum Blotter (Bio-Rad Laboratories) following the manufacturer's protocol and as previously described [16,28]. Southern blots were hybridised with labelled bla_{CMY-2} probe using Genius DIG labelling and detection kits (Roche) [17].

2.5. Plasmid transfer by conjugation

Conjugations were carried out as previously described [16]. Donor strains of *Salmonella* were PCR-positive for the bla_{CMY-2} gene conferring β-lactam resistance. The recipient strain of *S. enterica* serovar Typhimurium (JG798) was resistant to nalidixic acid for counterselection. Transconjugants were selected on Lauria–Bertani agar containing 100 µg/mL ampicillin and 20 µg/mL nalidixic acid. Susceptibility

Table 1
β-Lactamase-encoding genes and integrons detected by polymerase chain reaction

Gene	Forward primer	Reverse primer	No. of isolates positive (%)	Reference
bla_{CMY-2}	5′-GACAGCCTCTTTCTCCACA-3′	5′-TGGAACGAAGGCTACGTA-3′	102/125 (81.6%)	Gray et al. [17]
bla_{TEM-1}	5′-GGAAGAGTATGAGTATTC-3′	5′-CAGTTACCAATGCTTAATC-3′	2/125 (1.6%)	This work
bla_{SHV}	5′-GGTTATGCGTTATATTCGCC-3′	5′-TTAGCGTTGCCAGTGCTC-3′	3/125 (2.4%)	Rasheed and Tenover [26]
bla_{CTX-M} I	5′-GACGATGTCACTGGCTGAGC-3′	5′-AGCCGCCGACGCTAATACA-3′	0/125 (0%)	Pitout et al. [25]
bla_{CTX-M} II	5′-GCGACCTGGTTAACTACAATCC-3′	5′-CGGTAGTATTGCCCTTAAGCC-3′	0/125 (0%)	Pitout et al. [25]
bla_{CTX-M} III	5′-CGCTTTGCCATGTGCAGCACC-3′	5′-GCTCAGTACGATCGAGCC-3′	1/125 (0.8%)	Pitout et al. [25]
bla_{CTX-M} IV	5′-GCTGGAGAAAAGCAGCGGAG-3′	5′-GTAAGCTGACGCAACGTCTG-3′	0/125 (0%)	Pitout et al. [25]
intI1	5′-ACATGTGATGGCGACGCACGA-3′	5′-ATTTCTGTCCTGGCTGGCGA-3′	34/125 (27.2%)	This work
intI2	5′-CACGGATATGCGACAAAAGGT-3′	5′-GTAGCAAACGAGTGACGAAAATG-3′	3/125 (2.4%)	This work
intI3	5′-GCCTCCGGCAGCGACTTTCAG-3′	5′-ACGGATCTGCCAAACCTGACT-3′	0/125 (0%)	This work
intI4	5′-TTCAACGCTCGCAACTAGAAC-3′	5′-GTGTGGCAAGTCACGGTCTTT-3′	0/125 (0%)	This work

patterns of the transconjugants were determined as described in Section 2.1.

3. Results

3.1. Cephalosporin susceptibility of Salmonella isolated from animals

From 1999 to 2003, 34,411 *Salmonella* isolates were obtained from diagnostic sources ($n = 11,822$), on-farm studies ($n = 4059$), sampling at federally inspected slaughter facilities ($n = 17,539$) or other sources ($n = 991$). Isolates were assayed for susceptibility to 16 antimicrobials, 6 of which were *β*-lactam antibiotics, namely amoxicillin/clavulanic acid, ampicillin, cefalothin, cefoxitin, ceftiofur and ceftriaxone. Four of the antimicrobials were cephalosporins: a narrow-spectrum first-generation cephalosporin, cefalothin; an expanded-spectrum second-generation cephalosporin, cefoxitin; and two broad-spectrum 3GCs, ceftiofur (used in veterinary medicine) and ceftriaxone (used in human medicine) [29]. The minimum inhibitory concentrations for 50% and 90% (MIC_{50} and MIC_{90}) of all *Salmonella* in this study and the percent resistance to the *β*-lactams was determined (Table 2). Due to ceftiofur use in animals, this study focused on *Salmonella* resistance to this 3GC as determined by the CSLI breakpoint [23]. The prevalence of ceftiofur
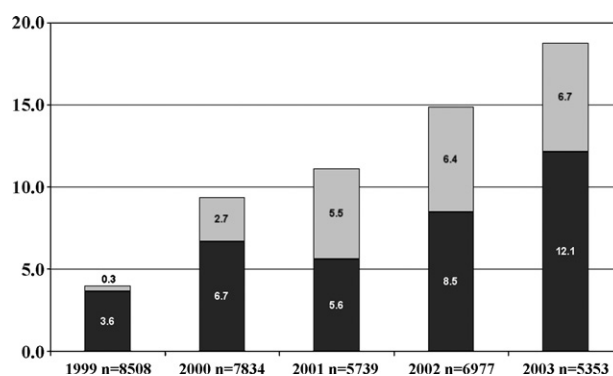

Fig. 1. Total percentage of *Salmonella* animal isolates resistant to the third-generation cephalosporin ceftiofur from 1999 to 2003 (n = total number of *Salmonella* isolated each year). The contribution of *Salmonella enterica* serovar Newport to the total ceftiofur-resistant *Salmonella* isolates is indicated by the grey bars and black numbers; the total of all other *Salmonella* serovars is indicated by black bars and white numbers.

resistance was 10.9% (3749/34,411) of *Salmonella* isolated from animals over the 5-year period. The percentage resistance rose over this period from 4.0% in 1999 (337/8508) to 18.8% (1005/5353) in 2003 (Table 2; Fig. 1). The percentage of ceftiofur resistance increased at a linear rate as indicated by the Pearson correlation coefficient ($r = 0.991$). However, resistance to ceftriaxone, the 3CG used in human medicine, remained low at ca. 0.3% throughout the study period (Table 1).

Table 2
Susceptibility of *Salmonella enterica* animal isolates to β-lactams, 1999–2003

Antimicrobial	1999 ($n = 8508$)			2000 ($n = 7834$)			2001 ($n = 5739$)			2002 ($n = 6977$)			2003 ($n = 5353$)		
	MIC_{50}	MIC_{90}	%R^a	MIC_{50}	MIC_{90}	%R^a	MIC_{50}	MIC_{90}	%R^a	MIC_{50}	MIC_{90}	%R^a	MIC_{50}	MIC_{90}	%R^a
Amoxicillin/clavulanic acid	1	16	3.6	1	16	9.7	1	>32	12.1	≤1	>32	15.5	≤1	>32	18.8
Ampicillin	≤2	>32	18	≤2	>32	23.7	≤2	>32	27.6	2	>32	28.5	≤1	>32	29.8
Cefalothin (1GC)	2	8	5.3	2	32	11.0	2	>32	13.6	4	>32	16.2	4	>32	20.5
Cefoxitin (2GC)	N.D.	N.D.	N.D.	≤4	16	9.4	≤4	32	10.9	2	>16	13.6	2	>16	16.4
Ceftiofur (3GC)	≤0.5	1	4.0	≤0.5	2	9.3	≤0.5	16	11.1	0.5	>8	14.9	0.5	>8	18.8
Ceftriaxone (3GC)	≤0.25	≤0.25	0.2	≤0.25	≤0.25	0.2	≤0.25	8	0.2	≤0.25	8	0.3	≤0.25	16	0.3

$MIC_{50/90}$, minimum inhibitory concentrations (in μg/mL) for 50% and 90% of all *Salmonella*; 1GC, narrow-spectrum first-generation cephalosporin; 2GC, expanded-spectrum second-generation cephalosporin; N.D., not determined; 3GC, broad-spectrum third-generation cephalosporin.

[a] Percentage resistance based on Clinical and Laboratory Standards Institute breakpoints.

The vast majority of ceftiofur-resistant *Salmonella* were also resistant to other antimicrobials (data available at http://www.ars.usda.gov/Main/docs.htm?docid=6750). Of the 3749 ceftiofur-resistant isolates, 39 (1.0%) were resistant to two or fewer other antimicrobials, 920 (24.5%) were resistant to between three and seven additional antimicrobials and 2790 (74.4%) were resistant to eight or more other antimicrobials. The most prevalent MDR phenotype for cephalosporin-resistant *Salmonella* isolates included resistance to the β-lactams (amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, cefalothin) as well as to chloramphenicol, streptomycin, sulfamethoxazole and tetracycline. This pattern was observed for 928/3749 (24.8%) of the ceftiofur-resistant *Salmonella* isolates.

3.2. Prevalence of ceftiofur-resistant Salmonella in animal sources

Ceftiofur-resistant *Salmonella* were recovered from all agricultural animal sources represented in the study, including cattle (beef and dairy), poultry (turkeys, chickens and chicken eggs) and swine (Table 3). The total *Salmonella* cattle isolates resistant to ceftiofur, including those not classified as beef or dairy, was 2092/11,915 (17.6%). *Salmonella* isolated from all cattle sources were significantly more likely to be ceftiofur resistant than the average of 10.9% as determined by the χ^2 test ($P < 0.0001$). Dairy cattle demonstrated the highest level of resistance (28.3%; 1011/3570), whilst ceftiofur resistance in those classified as beef cattle isolates was 19.5% (279/1428) (Table 3). *Salmonella* poultry isolates were significantly less ceftiofur resistant than the average, including

chickens (7.1%; 543/7697) and turkeys (6.8%; 213/3123). *Salmonella* isolated from chicken eggs were least likely to show ceftiofur resistance (3.6%; 28/783) and isolates from swine were also less likely to exhibit resistance to this 3GC (4.6%; 318/6942).

Companion animals and horses also yielded *Salmonella* isolates resistant to ceftiofur. The number of resistant *Salmonella* isolated from horses (19.2%; 249/1300) was significantly higher than average. No significant difference in carriage of ceftiofur-resistant *Salmonella* was seen for cats (9.8%; 13/133), whilst *Salmonella* isolated from dogs exhibited significantly higher levels of resistance (20.8%; 87/418). There were also 2100 *Salmonella* isolates from various other animal sources, including exotic agricultural animals (ostrich, gnu, alpaca, llama, etc.), pets (reptiles, bird, etc.), wild animals (otter, sea lion, deer, lizards, bear, etc.), zoological park animals (primates, large cats, zebra, etc.) and others, which overall did not exhibit significantly higher levels of ceftiofur resistance than average (9.8%; 206/2100).

3.3. Distribution of resistant Salmonella among clinical sources

Clinical status of isolates included diagnostic isolates obtained from specimens submitted to a veterinary diagnostic laboratory, on-farm isolates from apparently healthy animals and slaughter isolates obtained from federally inspected slaughter and processing plants (Table 3). Overall, diagnostic isolates were almost twice as likely to be ceftiofur resistant (18.5%; 2186/11,822). On-farm isolates presumed to originate from healthy animals had lower levels of

Table 3
Most prevalent host animal sources of ceftiofur-resistant *Salmonella* isolates

Host animal	All isolates		Diagnostic isolates		On-farm isolates		Slaughter isolates		All other isolates	
	Total	No. (%) R^b	Total	No. (%) R^b	Total	No. (%) R^b	Total	No. (%) R^b	Total	No. (%) R^b
Cattle										
Cattle[a]	6,917	802 (11.6)	1,647	291 (17.7)	706	64 (9.1)	4,564	447 (9.8)	–	–
Beef	1,428	279 (19.5)[c]	420	104 (24.8)	–	–	1,008	175 (17.4)	–	–
Dairy	3,570	1011 (28.3)[c]	2,782	997 (35.8)	788	14 (1.8)	–	–	–	–
Total cattle	11,915	2092 (17.6)[c]	4,849	1392 (28.7)	1494	78 (5.2)	5,572	622 (11.2)	–	–
Poultry										
Turkey	3,123	213 (6.8)[d]	806	121 (15.0)	–	–	2,287	90 (3.9)	30	2 (6.7)
Chicken	7,697	543 (7.1)[d]	550	36 (6.5)	49	3 (6.1)	6,585	484 (7.4)	513	20 (3.9)
Egg	783	28 (3.6)[d]	–	–	–	–	743	4 (0.5)	40	24 (60.0)
Swine	6,942	318 (4.6)[d]	2,694	211 (7.8)	1913	54 (2.8)	2,335	53 (2.3)	–	–
Horse	1,300	249 (19.2)[c]	1,258	249 (19.8)	42	0 (0.0)	–	–	–	–
Companion animals										
Cat	133	13 (9.8)	133	13 (9.8)	–	–	–	–	–	–
Dog	418	87 (20.8)[c]	418	87 (20.8)	–	–	–	–	–	–
Others	2,100	206 (9.8)	1,114	77 (6.9)	561	2 (0.4)	17	1 (5.9)	408	126 (30.9)
Totals	34,411	3749 (10.9)	11,822	2186 (18.5)	4059	137 (3.4)	17,539	1254 (7.1)	991	172 (17.4)

[a] Includes both dairy and beef cattle.
[b] Number (percentage) of isolates resistant to the third-generation cephalosporin ceftiofur.
[c] Animal groups with significantly higher levels of resistance to the third-generation cephalosporin ceftiofur, by χ^2 test ($P < 0.0001$).
[d] Animal groups with significantly lower levels of resistance to the third-generation cephalosporin ceftiofur, by χ^2 test ($P < 0.0001$).

resistance (3.4%; 137/4059), whilst slaughter isolates also showed lower resistance but closer to the average at 7.1% (1254/17,539). Although most diagnostic isolates had generally higher levels of ceftiofur resistance, levels in cattle, horses and dogs were noticeably higher. The percentage resistance in *Salmonella* from all diagnostic cattle isolates was 28.7% (1392/4849), whilst the levels in beef cattle were 24.8% (104/420) and in dairy cattle 35.8% (997/2782). The fraction of resistant horse clinical isolates was also high, with all resistant isolates coming from clinical samples (19.8%; 249/1258) and none coming from the 42 on-farm samples. The same was true for cats and dogs, as all samples for these animals were clinical isolates. *Salmonella* isolated from on-farm samples had universally lower levels of cephalosporin resistance for all animals (Table 3). Likewise, all slaughter isolates had lower than average levels of ceftiofur resistance, except for beef cattle (17.4%; 175/1008).

3.4. Distribution of resistance among Salmonella serotypes

Of the more than 2400 serotypes described for *S. enterica*, 234 have been submitted to the animal arm of NARMS. From 1999 to 2003, 215 of these serotypes were detected; 76 had a ceftiofur-resistant isolate. Of these, 42 serotypes with more than 100 isolates in total over the 5-year period are listed in Table 4 in order of their contribution to the total amount of cephalosporin-resistant *Salmonella*. Five serotypes, Newport, Typhimurium (including variant Copenhagen, now reported as var 5-), Agona, Heidelberg and Kentucky, accounted for almost 80% of ceftiofur-resistant *Salmonella*. Eight serotypes were significantly more resistant to ceftiofur than the average for all *Salmonella* (Table 4). Most of these also affected the overall *Salmonella* resistance, with serotype Newport contributing 36.2% and Typhimurium 23.5% of all resistant isolates. In addition, 20 serotypes were determined to be significantly less likely to be ceftiofur resistant, including Kentucky (4.2%; 131/3124) and Heidelberg (7.8%; 240/3079), which are the two most prevalent serotypes reported in NARMS animal isolates.

Salmonella Newport isolates exhibited a high percentage of resistance to ceftiofur (70.4%) and contributed substantially (36.2%) to total *Salmonella* resistance observed during the study. The percentage resistance and the fraction of total *S.* Newport resistant to ceftiofur for each year was 20.9% (28/134) in 1999, 74.8% (211/282) in 2000, 69.2% (315/455) in 2001, 77.9% (447/574) in 2002 and 73.7% (356/483) in 2003. The proportion of ceftiofur-resistant *Salmonella* that were *S.* Newport for each year was 8.3% (28/337) in 1999, 28.8% (211/732) in 2000, 49.5% (315/637) in 2001, 43.1% (447/1038) in 2002 and 35.4% (356/1005) in 2003. This indicated that serotype Newport was responsible for nearly one-third to one-half of the ceftiofur-resistant *Salmonella* isolated in some years and significantly contributed to the increase in resistance observed during the study (Fig. 1).

3.5. Detection of resistance genes and integrons in ceftiofur-resistant Salmonella isolates

From the ceftiofur-resistant *Salmonella* collected during the study, 125 representatives of prevalent serotypes, clinical sources and animals were selected for molecular analysis. To identify some of the genetic element(s) responsible for ceftiofur resistance, PCR assays for several β-lactamase-encoding genes (bla_{CMY-2}, bla_{TEM}, bla_{SHV} and bla_{CTX-M} groups I–IV) were performed [17,25,26,30]. The majority of the isolates assayed (81.6%; 102/125) were PCR-positive for the bla_{CMY-2} gene. Two of these were also bla_{TEM}-positive. Only 3 of the 125 isolates gave a positive PCR result for bla_{SHV}; 1 of these was also bla_{CMY-2}-positive. A single isolate was positive both for the bla_{CTX-M} group III allele and bla_{CMY-2}. The remaining 21 isolates (16.8%) did not harbour any of these β-lactamase genes. All PCR assays for class 3 and 4 integrons were negative. Three isolates were positive for class 2 integron, whilst 34 (27.2%) of 125 isolates were PCR-positive for the class 1 integron *intI1* integrase gene. Thirty of the *intI1*-positive isolates were also positive for bla_{CMY-2}.

3.6. Plasmid analysis of ceftiofur-resistant Salmonella isolates

Gel electrophoresis of plasmid DNA extracts detected plasmids in all of the 125 ceftiofur-resistant isolates tested. These plasmids ranged in size from 2.5 kb to >200 kb, and most isolates had multiple plasmids. All isolates had plasmids between 100 kb and 220 kb (Fig. 2(A)). Southern blot analysis of plasmids indicated that large plasmids carrying the bla_{CMY-2} gene were present in the isolates shown (Fig. 2(B)). All bla_{CMY-2} PCR-positive isolates analysed by Southern blot demonstrated hybridisation to one plasmid in each isolate ranging in size from ca. 50 kb to >220 kb, with the majority estimated to be ca. 180–220 kb (data not shown).

3.7. Transfer of plasmids encoding ceftiofur resistance by conjugation

Ceftiofur-resistant donors were mated in vitro with a sensitive recipient strain, and transconjugants were isolated after mating by plating on selective media. Transfer of plasmid DNA was confirmed by gel analysis of the transconjugants' plasmids, and Southern blot detection confirmed the transfer of the bla_{CMY-2} gene encoded on the plasmid (Fig. 3). This demonstrated that the large ca. 220 kb plasmid encoding cephalosporin resistance was transferred by conjugation. Resistance to ceftiofur in the transconjugants was confirmed by susceptibility testing. Additionally, many donor strains were MDR and the transfer of many other resistance traits was confirmed by susceptibility testing of the transconjugants (data not shown).

Table 4
Serotypes of *Salmonella enterica* with resistance to the third-generation cephalosporin ceftiofur and with more than 100 animal isolates from 1999 to 2003

Salmonella enterica (serotype)[a]	Serogroup[a]	No. of isolates	% of all *Salmonella*	No. of resistant isolates	% Resistance for this serotype	% of all resistant *Salmonella*
Newport	C2	1928	5.6	1357	70.4[b]	36.2
Typhimurium var. Copenhagen (5-)	B	2950	8.6	484	16.4[b]	12.9
Typhimurium	B	2761	8.0	398	14.4[b]	10.6
Agona	B	1245	3.6	363	29.2[b]	9.7
Heidelberg	B	3079	8.9	240	7.8[c]	6.4
Kentucky	C3	3124	9.1	131	4.2[c]	3.5
Uganda	E1	311	0.9	119	38.3[b]	3.2
Reading	B	532	1.5	91	17.1[b]	2.4
Mono	B	679	2.0	41	6.0[c]	1.1
Dublin	D1	313	0.9	30	9.6	0.8
Bredeney	B	150	0.4	30	20.0[b]	0.8
Montevideo	C1	1918	5.6	28	1.5[c]	0.7
Derby	B	1426	4.1	25	1.8[c]	0.7
Infantis	C1	653	1.9	24	3.7[c]	0.6
Anatum	E1	1226	3.6	21	1.7[c]	0.6
Untypeable	unk	250	0.7	18	7.2	0.5
Oranienburg	C1	242	0.7	17	7.0	0.5
Newbrunswick	E2	107	0.3	17	15.9[b]	0.5
Hadar	C2	1034	3.0	16	1.5[c]	0.4
Non-motile	unk	136	0.4	16	11.8	0.4
Thompson	C1	485	1.4	15	3.1[c]	0.4
Brandenburg	B	205	0.6	13	6.3	0.3
Senftenberg	E4	842	2.4	12	1.4[c]	0.3
Muenster	E1	815	2.4	12	1.5[c]	0.3
Saintpaul	N	266	0.8	11	4.1	0.3
Ohio	C1	166	0.5	11	6.6	0.3
Enteritidis	D1	706	2.1	10	1.4[c]	0.3
Cerro	K	374	1.1	9	2.4[c]	0.2
Muenchen	C1	281	0.8	9	3.2[c]	0.2
Tennessee	C1	131	0.4	9	6.9	0.2
Mbandaka	C1	630	1.8	7	1.1[c]	0.2
Schwarzengrund	B	490	1.4	6	1.2[c]	0.2
Johannesburg	R	234	0.7	6	2.6[c]	0.2
Branderup	C1	212	0.6	6	2.8	0.2
Choleraesuis var. Kunzendorf	C1	881	2.6	5	0.6[c]	0.1
Give	E1	189	0.5	5	2.6	0.1
Worthington	G2	169	0.5	5	3.0	0.1
Arizonae	S	345	1.0	4	1.2[c]	0.1
London	E1	112	0.3	2	1.8	0.1
Istanbul	C3	104	0.3	2	1.9	0.1
Meleagridis	E1	402	1.2	1	0.2[c]	<0.1
Litchfield	C2	102	0.3	1	1.0	<0.1

unk, unknown.

[a] Determined using the Kaufmann–White antigenic scheme.

[b] Serotypes with significantly higher levels of resistance to ceftiofur by χ^2 test ($P < 0.0001$).

[c] Serotypes with significantly lower levels of resistance to ceftiofur by χ^2 test ($P < 0.0001$).

4. Discussion

The incidence of resistance to the 3GC ceftiofur in *Salmonella* isolated from animals increased from 1999 to 2003 (Table 2; Fig. 1). However, resistance in human *Salmonella* isolates did not increase to levels seen in animals and was reported to be 2.1% in 1999, 3.2% in 2000, 4.1% in 2001, 4.3% in 2002 and 4.5% in 2003 (http://www.cdc.gov/narms/annual/2003/NARMS2003AnnualReport.pdf). In addition, resistance to ceftiofur increased at a slower rate in human isolates and demonstrated a weaker linear relationship (Pearson correlation coefficient $r = 0.939$) compared

with animal isolates ($r = 0.991$). Moreover, because both the human and animal NARMS are passive systems, a cause and effect relationship cannot be established between the animal and human arms of the system.

The distribution of ceftiofur-resistant *Salmonella* isolated from specific animals from 1999 to 2003 differed compared with the 1997–1998 report [17]. Previously, isolates from turkeys, horses, cats and dogs were more likely to be resistant to ceftiofur, whereas this study identified cattle, horse and dog isolates as significantly more likely to be ceftiofur resistant. In addition, the current study also found that resistance was significantly higher in isolates from
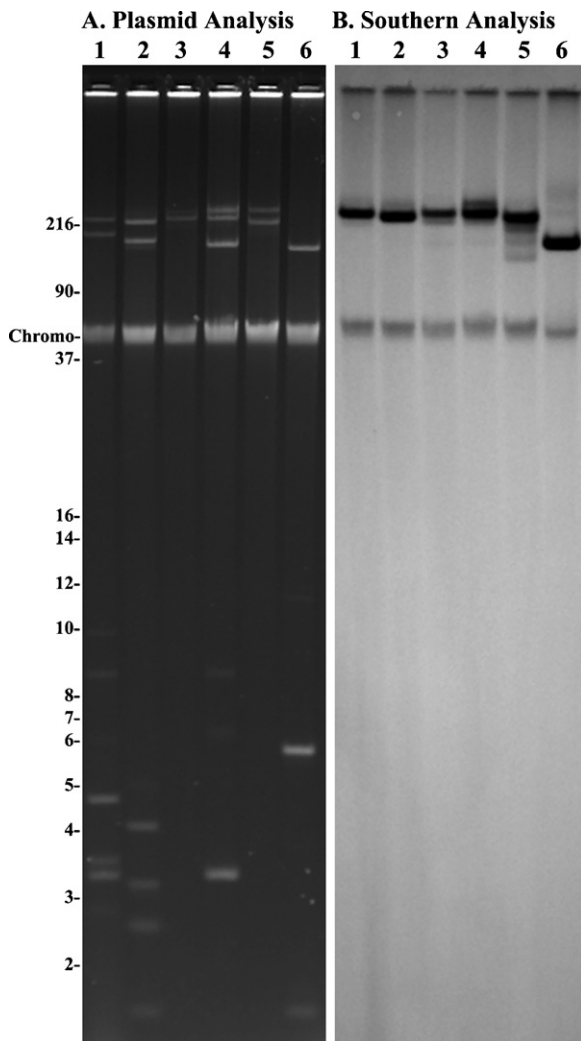
Fig. 2. Plasmid analysis and Southern hybridisation of ceftiofur-resistant *Salmonella* isolates from animal sources. (A) Plasmid DNA extracted from *Salmonella* isolates and separated on a 0.6% agarose gel. Plasmid molecular weights and chromosomal DNA are indicated on the left. Lane 1, *S.* Typhimurium JF200; lane 2, *S.* Montevideo JF209; lane 3, *S.* Heidelberg JF208; lane 4, *S.* Heidelberg JF210; lane 5, *S.* Heidelberg JF213; and lane 6, *S.* Derby JF207. (B) Southern transfer of the gel in (A) probed with digoxigen-labelled *bla*$_{CMY-2}$ polymerase chain reaction product.

diagnostic laboratories than from slaughter or on-farm samples. It is not surprising that isolates recovered from diagnostic submissions would be more resistant. For example, cattle are high-value animals and often receive antimicrobial therapy such as cephalosporins and other *β*-lactams to treat respiratory disease, mastitis and lameness [31]. Isolates originating from these animals may have acquired resistance due to treatment for diseases or exposure to other sick and treated animals. This is borne out by generally lower amounts of resistant *Salmonella* isolated from healthy cattle sampled on-farm and at the slaughter houses. Some other animals also had lower levels of ceftiofur-resistant isolates on-farm, including turkeys, chickens, eggs and swine. As with cattle, *Salmonella* from turkeys and swine were also more resistant from clinical submissions compared with on-farm and slaughter isolates.
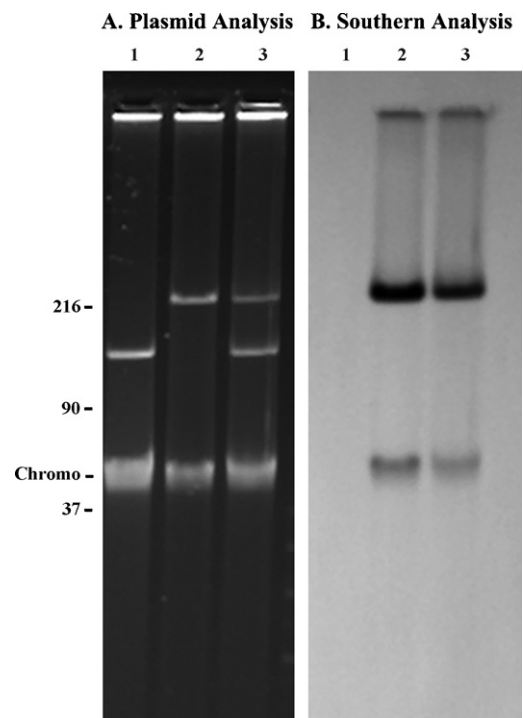

Fig. 3. Plasmid analysis and Southern hybridisation of strains from conjugation experiment. (A) Plasmid DNA extracted from *Salmonella* isolates separated on 0.6% agarose gel. Lane 1, JG798 (conjugation recipient); lane 2, JG1198 (multidrug-resistant donor); and lane 3, JF217 (ceftiofur-resistant transconjugant). Plasmid molecular weights and chromosomal DNA are indicated on the left. (B) Southern blot of gel in (A) hybridised with labelled *bla*$_{CMY-2}$ polymerase chain reaction product.

However, the levels of resistant isolates from chickens did not vary greatly between clinical, on-farm or slaughter sources, which could be a reflection of production practices or the relatively short grow-out time.

The proportion of ceftiofur-resistant isolates for each *Salmonella* serotype varied widely. The problem of MDR ceftiofur-resistant *S.* Newport is well documented by several studies and has been linked to a multidrug resistance plasmid-borne *bla*$_{CMY-2}$ gene [32–34]. It is unclear whether the increase in resistant *S.* Newport is due to expansion of a resistant *S.* Newport clone or the spread of a *bla*$_{CMY-2}$-encoding plasmid. Several recent studies have implicated clonal expansion as a likely cause [33,35,36] and analysis of isolates from our study is underway. Other serotypes also had different levels of resistance, including Typhimurium, which was the most ceftiofur-resistant serotype in the previous report [17] and number two in this report. Rarely isolated serotypes, including Uganda and Bredeney, also appeared to have high levels of ceftiofur resistance. Conversely, some serotypes, such as Kentucky, exhibited significantly lower levels of ceftiofur resistance. Interestingly, whilst Kentucky is the most prevalent serotype isolated from animals, it is rarely isolated from humans (Table 4). Other serotypes are frequently isolated both from animals and humans, such as *S.* Enteriditis, which is second in human and fourteenth in

animal prevalence. Notably, *S.* Enteriditis isolated from animals has a very low level of ceftiofur resistance (2.1%) (Table 4). These data suggest that the relationship between serotype, animal host and resistance is complex and requires further investigation.

The large impact of *S.* Newport on the total number of resistant *Salmonella* isolated during this study demonstrates that trends in total resistance can be misleading and underscores the necessity of detailed analysis of serotype, host species and clinical status to understand what may be affecting *Salmonella* prevalence and resistance. In the case of resistant *S.* Newport, it was found to be associated with cattle and especially clinically ill cattle. A recent study has associated human infections by resistant *S.* Newport with beef products as well as chicken eggs [34]. However, only 40 *Salmonella* of 7697 isolated from chickens during this study were serotype Newport and none were isolated from eggs. Overall, this suggests that the increase in ceftiofur-resistant *Salmonella* may be predominantly attributed to serotype Newport from cattle.

The genetic element responsible for most of the ceftiofur resistance in *Salmonella* isolated from US animals appears to be the bla_{CMY-2} gene. As with the previous study, this was found to be true for all serotypes tested. The gene appears to be encoded on a large plasmid between 100 kb and 200 kb and has been shown to be mobilised by conjugation [17,37]. These also appear to be multidrug resistance plasmids and transfer resistance phenotypes to as many as 13 antimicrobial compounds to recipients. Plasmids isolated from MDR *S.* Newport are being investigated by several groups; analysis may aid in determining whether increasing cephalosporin resistance in *Salmonella* is due to the spread of these plasmids. However, the number of serotypes in which the bla_{CMY-2} gene is detected suggests that the plasmid is spreading to new serotypes at a detectable level.

Whilst very low numbers of ceftiofur-resistant animal isolates were positive for bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes, it is possible that some were not detected by the assays owing to divergence. Most of these other β-lactamase genes were co-resident with the bla_{CMY-2} gene, which has been described previously [38]. More importantly, ca. 17% of isolates had no β-lactamase gene detected by the PCR assays even though they were resistant to the 3GC ceftiofur (Table 1). This indicates that not only is the spread of the bla_{CMY-2} gene a concern, but that there are also other undetected resistance mechanisms associated with ceftiofur resistance in *Salmonella*. Additionally, the *intI1* gene was detected in 27.2% of the resistant isolates tested. The bla_{CMY-2} gene was also co-resident in most of these isolates (88.2%; 30/34). Studies are currently underway to find any linkage between the integrons and cephalosporin resistance genes.

The incidence of cephalosporin-resistant *Salmonella* isolated from humans has increased substantially in the rest of the world and to a lesser extent in the USA [18,19,30], and this study improves our knowledge of the development of resistance in animals. The animal species, clinical status and serotypes identified in this study with significantly higher levels of resistance to the 3GC ceftiofur warrant further investigation. Although the plasmid-borne bla_{CMY-2} gene is most likely the predominant cause of cephalosporin resistance in *Salmonella* isolated from animals in the USA, there are other unknown resistance mechanisms that should be explored. Analysis of these data is crucial in developing intervention strategies to safeguard animal and human health.

Acknowledgments

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